

Tyrosine: an inhibitor of LDL oxidation and endothelial cell cytotoxicity initiated by superoxide/nitric oxide radicals

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Abstract Tyrosyl radicals can catalyze LDL oxidation. In addition to their LDL oxidizing ability, superoxide ($O_2^{\cdot-}$)/nitric oxide (NO^{\cdot}) generate phenoxyl radicals when reacting with tyrosine. Therefore we tested if tyrosine can act as a pro-oxidant in $O_2^{\cdot-}$ / NO^{\cdot} -initiated LDL oxidation. When LDL was exposed to $O_2^{\cdot-}$ / NO^{\cdot} , tyrosine exerted a strong inhibitory effect on $O_2^{\cdot-}$ / NO^{\cdot} -initiated LDL oxidation as measured by TBARS formation and alteration in electrophoretic mobility of LDL. Tyrosine was also able to protect human endothelial cells from the cytotoxic effect of $O_2^{\cdot-}$ / NO^{\cdot} . Because $O_2^{\cdot-}$ / NO^{\cdot} can occur in vivo, the results may indicate that serum-free tyrosine could act as an efficacious physiological antioxidant in case of $O_2^{\cdot-}$ / NO^{\cdot} -initiated LDL oxidation and endothelial cell cytotoxicity.

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Key words: LDL oxidation; Superoxide; Nitric oxide; Antioxidant; Tyrosine; SIN-1

1. Introduction

There is experimental evidence that the oxidative modification of LDL plays a pathophysiological role in the onset of atherogenesis [1]. This observation has led to studies dealing with the inhibition of LDL oxidation by drugs or naturally occurring compounds [2–7]. Lipid peroxidation can be initiated by e.g. copper ions, organic peroxy radicals, hypochlorite and superoxide/nitric oxide radicals ($O_2^{\cdot-}$ / NO^{\cdot}) [8–11].

Recently, it has been shown that MPO in presence of H_2O_2 and tyrosine was able to generate tyrosyl radicals [12] which could act as catalysts for the initiation of LDL oxidation [13].

Tyrosyl radicals are also formed when the reaction product of $O_2^{\cdot-}$ / NO^{\cdot} reacts with free tyrosine or tyrosine residues in proteins resulting in chemical modification of the amino acid [14,15]. Taking this and the fact into account that $O_2^{\cdot-}$ / NO^{\cdot} can cause lipid peroxidation, one could assume that tyrosine may be able to act as a pro-oxidant in the $O_2^{\cdot-}$ / NO^{\cdot} -initiated LDL oxidation.

We report that tyrosine inhibited the $O_2^{\cdot-}$ / NO^{\cdot} -induced lipid peroxidation and, in addition, could protect human endothelial cells from the cytotoxic effect of $O_2^{\cdot-}$ / NO^{\cdot} radicals.

2. Materials and methods

Amino acids, 3-nitro-L-tyrosine and 3-morpholiniosydnonimine (SIN-1) were from Sigma Chemical Co. (St. Louis, MO).

2.1. LDL isolation

The isolation of LDL from human plasma followed procedures reported previously [16]. The final preparation was filter sterilized and stored in 0.15 mol/l NaCl containing 0.1 mmol/l EDTA.

2.2. Lipoprotein oxidation

LDL (2 mg/ml) was incubated in a total volume of 250 μ l (0.15 mol/l NaCl, 0.025 mol/l TRIS-HCl, pH 7.4) with or without SIN-1 for 18 h at 37°C. Lipid peroxidation products were measured as thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde equivalents (MDA) according to [17].

2.3. Agarose gel electrophoresis

Of the treated or untreated LDL, 10–20 μ g was analyzed on agarose gels using a commercial system (Lipidophor All In, Immuno AG, Vienna, Austria). Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation [1,8], setting the electrophoretic mobility of native (untreated) LDL arbitrarily as 1.

2.4. Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were prepared and cultured as previously reported [18]. Cells were seeded in 6-well culture plates. After cells had reached confluency the cells were washed with phosphate-buffered saline (PBS) and further cultured in a medium containing ions and glucose as given for RPMI-1640, but without amino acids.

2.5. Cytotoxicity

The release of lactate dehydrogenase activity (LDH) into the cell culture medium was taken as an indicator of cytotoxicity. LDH activity was measured by a commercial test kit (Boehringer Mannheim Automated Analysis for BM/Hitachi 717, Germany).

2.6. Analysis of reaction products

Reaction mixtures containing LDL (2 mg/ml) and the respective compounds (SIN-1, tyrosine, tryptophan) were analyzed by HPLC essentially as described by Kaur and Halliwell [19].

3. Results

3.1. LDL oxidation by superoxide/nitric oxide radicals

LDL was subjected to $O_2^{\cdot-}$ / NO^{\cdot} -induced lipid oxidation using the sydnonimine SIN-1, which generates simultaneously both radical species in solution at 37°C [10]. LDL was incubated for 18 h at 37°C with SIN-1 (up to 5 mmol/l) and subsequently the lipoprotein oxidation was measured by TBARS formation [10]. A concentration-dependent increase of TBARS was observed. However, in the presence of tyrosine (2 mmol/l) there were no TBARS formed, that is, no LDL oxidation occurred (see Fig. 1A).

In addition, agarose gel electrophoresis revealed, that the

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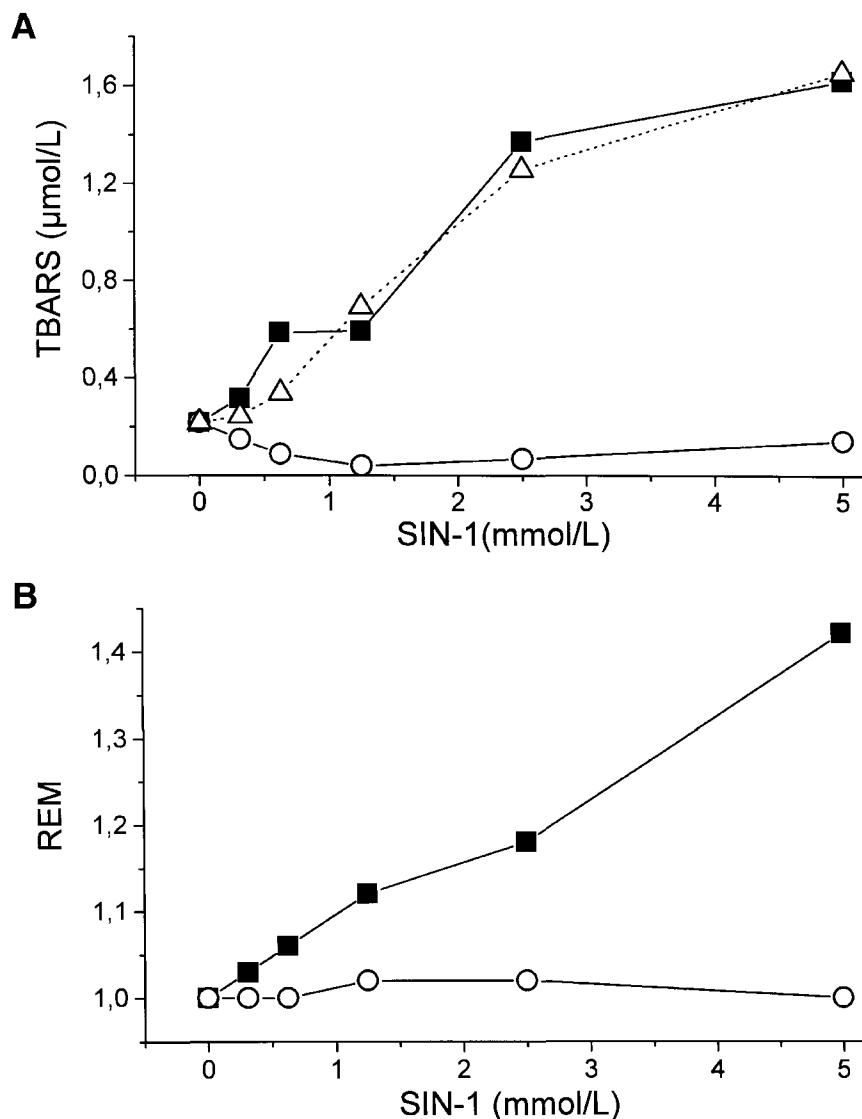


Fig. 1. A: Effect of tyrosine and phenylalanine on SIN-1-induced LDL oxidation. LDL (2 mg/ml) was incubated in the presence or absence of SIN-1 for 18 h at 37°C. Parallel incubations were run with tyrosine (2 mmol/l) or phenylalanine (2 mmol/l) and the respective SIN-1 concentration. LDL oxidation was expressed as TBARS formed as given in Section 2. ■, SIN-1; ○, SIN-1 and tyrosine; △, SIN-1 and phenylalanine. B: Effect of tyrosine on SIN-1-induced changes of LDL electrophoretic mobility. LDL (2 mg/ml) was incubated in the presence or absence of SIN-1 for 18 h at 37°C. Parallel incubations were run with tyrosine (2 mmol/l) and the respective SIN-1 concentration. The electrophoretic mobility relative to untreated LDL (REM) was determined as given in Section 2. ■, SIN-1; ○, SIN-1 and tyrosine.

SIN-1 induced increase in REM, which is a further indicator of LDL modification was completely inhibited in the presence of tyrosine (see Fig. 1B). Parallel incubations with phenylalanine (2 mmol/l) showed that this aromatic amino acid had no influence on the $O_2^{\cdot-}/NO^{\cdot}$ -induced lipid oxidation (see Fig. 1A).

Fig. 2 depicts the effect of increasing tyrosine concentrations on LDL oxidation caused by 1.25 mmol/l SIN-1. 50% inhibition of LDL oxidation was observed at about 125 μmol/l tyrosine. The well-known hydrophilic antioxidant ascorbic acid [20,21] was effective at about 3 times higher concentration.

The products of SIN-1 decomposition have been shown to lead to chemical modification, i.e. nitration of tyrosine [14,15]. Thus one may assume that tyrosine may act as a scavenger of the reactive SIN-1 decomposition products which in the absence of tyrosine oxidize the lipoprotein. Hence, already

modified tyrosine should possess less antioxidizing activity compared to unmodified tyrosine. As can be seen in Fig. 2, when 3-nitrotyrosine was added to the LDL oxidizing system, this compound was only effective at 2 mmol/l in inhibiting LDL oxidation by $O_2^{\cdot-}/NO^{\cdot}$. From all other amino acids tested (histidine, proline, tryptophan, cysteine; 2 mmol/l) tryptophan showed the highest inhibitory activity (66%) of LDL oxidation compared to tyrosine (86%). Cysteine known to be oxidized by peroxynitrite [22], a reaction product of SIN-1 decomposition, was less effective in inhibiting LDL oxidation (35% inhibition).

3.2. Endothelial cells

To test if tyrosine and other amino acids can also counteract the membrane lipid oxidizing, cell damaging effect of $O_2^{\cdot-}/NO^{\cdot}$ [23,24], HUVECs were exposed for 16 h to SIN-1 (1 mmol/l) and the respective amino acid. Cell morphology

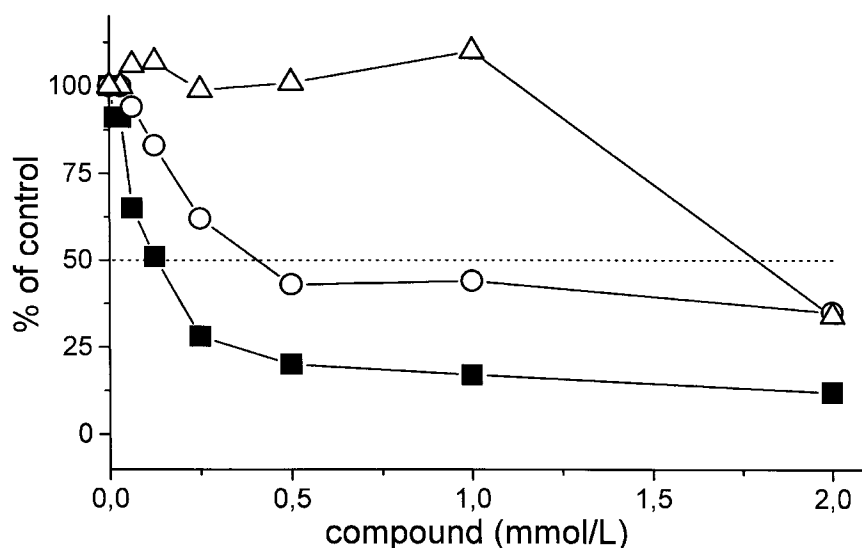


Fig. 2. Effect of tyrosine, 3-nitrotyrosine and ascorbic acid on SIN-1-induced LDL oxidation. LDL (2 mg/ml) was incubated with SIN-1 (1.25 mmol/l) for 18 h at 37°C in the absence or presence of the respective compound. ■, Tyrosine; △, 3-nitrotyrosine; ○, ascorbic acid.

(phase contrast microscopy) and LDH release were taken as indicators of cytotoxicity. SIN-1 treatment caused a release of LDH activity of 105 ± 2.0 U/l compared to 7.0 ± 3.1 U/l for untreated cells. Both, tyrosine and tryptophan when added to the culture medium (100 μ mol/l) protected the cells from the cytotoxic effect of $O_2^{\cdot-}/NO^{\cdot}$, that is, LDH release was diminished to near-control levels (see Table 1). No effect was seen when amino acids alone were added to HUVECs (data not shown). All other amino acids tested did not show a protective effect against the cell damaging potential of SIN-1 (Table 1).

Fig. 3 represents morphology of HUVEC monolayers after an 16 h incubation period with SIN-1 in the absence or presence of tyrosine and cysteine. SIN-1 led to retraction and subsequent detachment of endothelial cells, which was overcome by tyrosine, but not by cysteine.

Table 1
Effect of various amino acids on the SIN-1-induced cytotoxicity on HUVEC

Compound added	LDH(U/L)
none	7 ± 3.1
SIN-1	105 ± 2.0
SIN-1+tyrosine	31 ± 2.5
SIN-1+tryptophan	27 ± 0.6
SIN-1+threonine	122 ± 2.7
SIN-1+lysine	112 ± 5.9
SIN-1+cystine	95 ± 2.4
SIN-1+cysteine	129 ± 6.9
SIN-1+arginine	118 ± 3.1
SIN-1+serine	114 ± 2.7
SIN-1+phenylalanine	120 ± 7.4
SIN-1+histidine	140 ± 9.0
SIN-1+proline	144 ± 6.8

Effect of various amino acids on SIN-1-induced endothelial cell damage as measured by LDH release. HUVECs were exposed for 16 h to SIN-1 (1 mmol/l) in the absence or presence of amino acids (100 μ mol/l) at 37°C/5% CO_2 . The release of LDH was taken as an indicator of cytotoxicity and measured as given in Section 2. All experiments were done in triplicate. Means \pm SD are given.

4. Discussion

The present study suggests that tyrosine (tryptophan) is a potent antioxidant in the $O_2^{\cdot-}/NO^{\cdot}$ -induced oxidation of LDL. Simultaneously produced $O_2^{\cdot-}/NO^{\cdot}$ radicals may form peroxynitrous acid (ONOOH) or peroxynitrite (ONOO⁻), which can decompose to $\cdot OH$ and $\cdot NO_2$, capable of oxidizing tocopherol and lipids in LDL leading to the atherogenic alterations of the lipoprotein [25–27]. In addition to the lipid oxidizing ability of ONOOH, it is known that free tyrosine or tyrosine residues in proteins can be modified by ONOOH and ONOO⁻, forming nitrotyrosine [28,29]. Taking these observations into account, free tyrosine may act by scavenging the

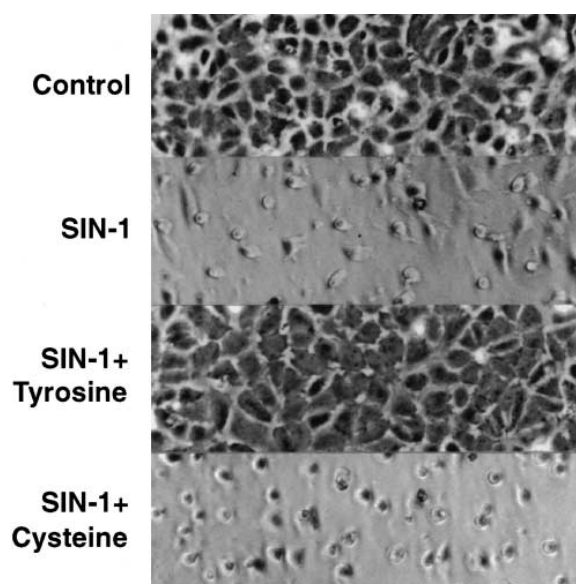


Fig. 3. Effect of tyrosine and cysteine on SIN-1 altered endothelial cell morphology. HUVECs were exposed for 16 h to SIN-1 (1 mmol/l) in the absence or presence of amino acids (100 μ mol/l) at 37°C/5% CO_2 . HUVEC monolayers as seen in the phase contrast microscope ($\times 100$ magnification) were photographed after 16 h of incubation in the respective medium.

reactive nitrogen and/or oxygen species, thus protecting LDL from oxidation. This suggestion was partially sustained by our results obtained with nitrotyrosine. In contrast to unmodified tyrosine, the nitrated amino acid showed very limited antioxidative potential, possibly due to the inability of the already nitrated amino acid to scavenge the lipid oxidizing reactive decomposition products of SIN-1. However, HPLC analysis of LDL/SIN-1/tyrosine and LDL/SIN-1/tryptophan incubation mixtures revealed that very low concentrations of nitrated amino acid derivatives were formed (results not shown), an observation which is in accordance with results of van der Vliet et al. [15]. These authors have shown that tyrosine in the presence of peroxynitrite or SIN-1 was converted to nitrotyrosine and dityrosine in low amounts. Thus, reaction mechanisms additional to tyrosine nitration may be responsible for the observed effect. The amino acid could scavenge radicals forming tyrosyl radicals [30] which can dimerize to dityrosine and in that way block the oxidative effect of $O_2^{\cdot-}/NO^{\cdot}$. On the other hand tyrosyl radical itself may be much less damaging than $O_2^{\cdot-}/NO^{\cdot}$. Phenylalanine, most related to tyrosine, was ineffective as antioxidant. Peroxynitrite can hydroxylate phenylalanine to tyrosine as reported by van der Vliet et al. [31] and one may speculate that during incubation of LDL/SIN-1/phenylalanine tyrosine may be generated and may inhibit the lipid peroxidation process. However, the actual amount of tyrosine formed by the reaction of phenylalanine with peroxynitrite is very low (5 mmol/l phenylalanine generate about 10 μ mol/l of tyrosines) [31]. This could explain the inability of phenylalanine to inhibit LDL peroxidation under the experimental conditions.

$O_2^{\cdot-}/NO^{\cdot}$ have been shown to oxidize membrane lipids, thus exerting cytotoxicity to endothelial cells [23]. Our results obtained with HUVECs show that tyrosine (tryptophan) was a potent cytoprotective agent for endothelial cells treated with $O_2^{\cdot-}/NO^{\cdot}$. Free tyrosine can reach plasma levels of about 100 μ mol/l [32], a concentration which was found to inhibit LDL oxidation and cytotoxicity towards endothelial cells by $O_2^{\cdot-}/NO^{\cdot}$ in our system.

In summary, our data indicate that tyrosine effectively counteracts the LDL modifying and cytotoxic effect of $O_2^{\cdot-}/NO^{\cdot}$. Both radical species can occur in vivo and the serum concentration of free tyrosine is fairly high, but further studies will be required to establish the in vivo antioxidant and cytoprotective effect of this amino acid.

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